UMO 1521.1
PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of James T. English, et al. Art Unit 1639
Serial No. 09/829,549
Filed April 10, 2001
Confirmation No. 8198
For PHAGE DISPLAY SELECTION OF ANTI FUNGAL PEPTIDES
Examiner Teresa D. Wessendorf

APPEAL BRIEF

Edward J. Hejlek, Reg. No. 31,525
SENNIGER POWERS
One Metropolitan Square, 16th Floor
St. Louis, Missouri 63102
(314) 231-5400

TABLE OF CONTENTS

TABLE OF AUTHORITIES

REFERENCES

CASES

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of James T. English, et al. Art Unit 1639
Serial No. 09/829,549
Filed April 10, 2001
Confirmation No. 8198
For PHAGE DISPLAY SELECTION OF ANTI FUNGAL PEPTIDES
Examiner Teresa D. Wessendorf

June 2, 2006

APPEAL BRIEF

This is an appeal from the final rejection of the claims of the above-referenced application made in the Office action dated July 25, 2005. A Notice of Appeal was filed on December 22, 2005.

The appeal brief fee in the amount of $250.00 and the fee for a three-month extension of time in the amount of $510.00 are submitted herewith.

I. REAL PARTY IN INTEREST

The real party in interest is The Curators of the University of Missouri, a corporation of the state of Missouri. An assignment of the above-identified application from the inventors to The Curators of the University of Missouri is recorded in the U.S. Patent and Trademark Office at Reel 011962, Frame 0536.

II. RELATED APPEALS AND INTERFERENCES

Appellants are unaware of any pending appeals or interferences which may be related to, directly affect or be affected by, or have a bearing on, the Board's decision in the present appeal.

III. STATUS OF CLAIMS

Claims 1-9 and 32-51 are pending in this application. The claims on appeal are set forth in full in the Claims Appendix of this Brief.

Claims 1-9 and 32-51 stand rejected under 35 U.S.C. §103(a). The rejection of claims 1-9 and 32-51 under 35 U.S.C. §103(a) is being appealed.

IV. STATUS OF AMENDMENTS

No amendments have been filed after the final rejection.

V. SUMMARY OF CLAIMED SUBJECT MATTER

Phytophthora is a disease-causing organism causing large losses in many agronomically important crop species. Control of these pathogens is particularly difficult, and often requires the treatment of entire fields with biocidal compounds. While such methods are generally effective, increasing concern about the environment and economic costs of such treatments require the need for alternative control methods. The claimed invention provides a solution to this problem: the use of random peptide display to identify peptides that bind to pathogenic fungi of the genus *Phytophthora* and other pathogenic fungi.[1]

More specifically, the present invention provides a method for identifying non-immunoglobulin peptides having an affinity for the surface of a plant pathogen. The method comprises, among other things, (a) constructing a library of peptides, (b) contacting a vector expressing the peptide library with a target fungus and removing unbound vector, (c) eluting bound vector from said fungus, (d) amplifying the bound vector, (e) sequencing the oligonucleotides contained in said eluted vector, (f) deducing the amino acid sequence of peptides encoded by said oligonucleotides contained in

[1] See Appellants' Specification, paragraph [0013].

said eluted vector, and (g) selecting the non-immunoglobulin peptides for which the amino acid sequence has been deduced.[2]

Practically speaking, the library of peptides can be displayed on a vector such as phage and incubated with fungi of the genus *Phytophthora* to select for binding peptides. As described in Example 3, the f8-1 or the f88-4 phage-displayed peptide library can be incubated with *Phytophthora* zoospores at room temperature with gentle agitation. Bound phage can then be eluted from the multitude of unknown epitopes on the *Phytophthora* zoospores with an elution buffer and purified. As described in Example 5, DNA can be isolated from phage clones that bound to the *Phytophthora* zoospores and sequenced to determine the peptide sequence that the DNA encodes.

After the binding peptides are identified by these steps, they can be assayed to determine their effect on *Phytophthora*. Importantly, the peptides identified in the examples were effective in inducing premature encystment of *Phytophthora* zoospores (see Appellants' Example 6). This is significant because premature encystment results in the disruption of the pathogenicity of the *Phytophthora* species. Thus, the peptides have an antifungal effect. In particular, the fraction of zoospores encysted by the Appellants' selected phage-bearing peptides was two to seven times greater than the fraction encysted by wild-type phage, as described in Example 6.

VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

The only issue presented on appeal is whether the subject matter of claims 1-9 and 32-51 satisfy the requirements of 35 U.S.C. §103(a).

VII. GROUPING OF CLAIMS

For purposes of this appeal, claims 1-9 and 32-51 do not stand or fall together. The claims have been divided into two groups: Group I (claims 1-8, 32-40, and 45-51) and Group II (claims 9 and 41-44). The claims of each of Groups I and II are separately and independently patentable for the reasons described in Sections VIII(A)(1-4) and VIII(B)(1-4), *infra*.

---

[2] See Appellants' claim 1.

VIII. ARGUMENT

A. The Group I Claims

Claim 1 is representative of the Group I claims. It is directed to a method for identification of **non-immunoglobulin** peptides having an affinity for the surface of a fungus. The method comprises (a) constructing a **library of peptides** by (i) preparing random oligonucleotides, (ii) inserting said oligonucleotides into a vector that expresses peptides encoded by said random oligonucleotides on its surface and is capable of transfecting a host cell, and (iii) transfecting a host cell with said vector to amplify said vector in an infectious form to create a library of peptides on the surface of said vector; (b) contacting said vector expressing said peptide library with a target fungus and removing unbound vector; (c) eluting bound vector from said fungus; (d) amplifying said bound vector; (e) sequencing the oligonucleotides contained in said eluted vector; (f) deducing the amino acid sequence of peptides encoded by said oligonucleotides contained in said eluted vector; and (g) selecting the **non-immunoglobulin peptides** for which the amino acid sequence has been deduced.

1. Gough et al.

Gough et al. describe methods for the isolation of antibodies specific for surface-exposed epitopes on certain species of *Phytophthora* to be used for production of immunological probes and single-chain Fv (scFv) antibodies.[3] Gough et al.'s method involves adding germlings and soluble components thereof to maxisorb immunotubes, blocking, and incubating the maxisorb immunotubes with a **phage-displayed antibody library**.[4] The nonbound phage is removed, and the bound phage eluted and amplified in *E. coli*.[5] These steps are repeated, resulting in a discrete population of phage antibody fragments.[6] The diversity of the eluted antibodies is established by *Bst*NI fingerprinting, and DNA encoding for selected single-chain Fv antibodies is amplified by PCR, digested with restriction endonucleases, and ligated into a vector to produce scFv

---

[3] Gough et al. at page 98.
[4] *Id.* at page 99.
[5] *Id.*; *see also* pages 101-102.
[6] *Id.*

fusion proteins.[7] The scFv fusion proteins are then used to recognize external epitopes of *Phytophthora*.[8]

Because many of the fungi used to asses the binding of the isolated single-chain Fv antibodies did not produce germlings that adhered to plastic surfaces, the antibodies isolated by Gough et al. had to be tested for their binding to mycelial homogenates.[9] According to Gough et al., however, this assay could not be performed with phage-displayed single-chain Fv's because the phage-displayed form of the antibodies exhibited high background binding to the mycelial homogenates, which necessitated the production of soluble single-chain Fv's.[10] Unfortunately, Gough et al. were also unable to produce detectable amounts of soluble single-chain Fv antibodies from the DNA sequences encoding the selected antibodies.[11] Accordingly, the scFv genes had to be subcloned into an expression vector for the production of a fusion protein containing the single-chain Fv protein and a maltose binding protein (MBP).[12]

While the methods of Gough et al. were generally effective in identifying antibodies that bind to the surface of *Phytophthora*, Gough et al. admit that their antibodies and others identified to date **have had no effect on *Phytophthora* whatsoever**. Gough et al. still promise, however, that other antibodies to surface epitopes of *Phytophthora* having a pathogenic effect could still be identified:

> The panning of whole pathogens might be expected to yield scFvs that bind to unmodified surface antigens that may be of importance in the infection process. However, preliminary assays, in which sporangia were mixed with soluble MBP-scFv fusion protein and then used to inoculate tomato leaf discs (Niderman et al., 1995), showed no detectable anti-fungal activity for any of the antibodies. Nevertheless, the isolation of other scFvs specifically directed against the native conformation of surface-accessible antigens may well provide new tools to probe and manipulate pathogenicity.[13]

---

[7] Gough et al. at pages 98-99; *see also* pages 102-103.
[8] *Id.* at page 100; *see also* pages 103-104.
[9] *Id.* at page 106.
[10] *Id.*
[11] *Id.*
[12] *Id.*
[13] *Id.* at 107.

In contrast to the methods of Gough et al., claim 1 requires the use of a library of non-immunoglobulin peptides, not a library of scFv antibody fragments. Because Gough et al. are concerned with the isolation of antibodies for the surface-exposed epitopes on certain *Phytophthora* species to be used for immunological probes, they are using only single-chain Fv antibody fragments on phage in their disclosed phage display methods, and report no problems with the use of such antibody fragments for their objectives. Significantly, therefore, not only does Gough et al. fail to teach or suggest the use of vector-displayed random peptide libraries in their methods, they also fail to teach or suggest the selection of non-immunoglobulin peptides that bind epitopes on the surface of a fungus.[14] Furthermore, the substitution of random peptide libraries for antibody fragment libraries would be unproductive as Gough et al. seek to identify antibodies which can be used in immunological methods, not mere non-immunoglobulin peptides.[15]

In general, the single-chain Fv antibodies utilized in the methods of Gough et al. are far more complex than the simple peptides in the library of claim 1. Single-chain Fv antibodies such as those utilized in the methods of Gough et al. consist of three independent variable regions of amino acids that are constrained in some fashion (e.g., by folding) by the remaining scFv scaffold. Consequently, the three regions can interact with a target in a constrained manner that is complex and difficult to predict. The scFv scaffold itself may also interact with the target in some manner. Stated another way, the active binding portion of the antibody fragment may or may not work by itself upon subsequent isolation, and/or molecular structures and components other than the active binding portion may be responsible for binding epitopes or surface functions on the target independently or in conjunction with the active binding portion.

The random peptide libraries of claim 1 have far greater utility since they simply have some level of affinity to cell surface factors on the target fungus, without the additional ancillary protein sequences involved in the complex aspects of antibody

---

[14] See Appellants' Specification, paragraph [0043] ("A "non-immunoglobulin peptide" means a peptide which is not an immunoglobulin, a recognized region of an immunoglobulin, or contains a region of an immunoglobulin. For example, a single chain variable region of an immunoglobulin would be excluded from this definition.").

[15] In re Gordon, 733 F.2d 900, 221 USPQ2d 1125 (Fed. Cir. 1984); M.P.E.P. §2143.01. As stated by the Federal Circuit, if proposed a modification would render the prior art unsatisfactory for its intended purpose, then there is no suggestion or motivation to make the proposed modification.

structure and form. That is, the selected non-immunoglobulin peptides of Appellants' claimed invention can work outside of or independently from a phage-antibody framework to provide a more direct interaction with its targets.

A person of skill in the art must ignore the express teachings of Gough et al. to arrive at a non-immunoglobulin approach using simple peptide libraries. Most significantly, Gough et al.'s approach did not work, i.e., it "showed no detectable anti-fungal activity for any of the antibodies," and, at best, they hold out some vague hope for the future but this hope was limited to scFvs and NOT non-immunoglobulin peptides. Thus, a person of skill in the art would not and could not be motivated to substitute a library of peptides for antibodies in the methods of Gough et al. for the selection of non-immunoglobulin peptides.

The Office vaguely asserts that "the suggested teachings of Gough of non-macromolecular species (i.e., fragments) that retain the recognition characteristics of antibodies like small molecule peptides mimics [*sic*], would suggest the claimed peptide."[16] In fact, Gough et al. teaches and emphasizes the use of antibodies and only antibodies, not simply "species that retain the recognition characteristics of antibodies" as asserted by the Office. In sharp contrast, the Appellants' invention functions independently of immunological response (i.e., non-immunoglobulin) and is claimed as such.[17] Just because you happen to have a peptide that binds to an epitope does not mean that it is an "antibody mimic," as the Office is apparently asserting.

Finally, the Office notes that Gough et al. refer to similar strategies that have been applied to the selection of phage displayed peptides that bind to the surface of intact platelets.[18] This reference by Gough et al., however, implies that mere peptides would be inadequate to accomplish the clearly-stated objectives of Gough et al.

2. Kodadek

Kodadek describes methods of isolating small peptides that recognize **specific, known target peptides** (i.e., target peptides the identity and sequence of which are already known) for use in affinity purification. Kodadek's methods involve an elaborate genetic selection scheme to identify certain library encoded peptides having affinity to a

---

[16] Office action mailed July 25, 2005, at page 3.
[17] *See* Appellants' claim 1 *and* Appellants' Specification, paragraph [0043].
[18] Office action mailed July 25, 2005, at page 4.

single known target peptide (i.e., not a multitude of unknown epitopes such as those on the surface of a fungus).

Briefly, Kodadek's genetic selection scheme involves forming two compatible constructs, one encoding the known target peptide, and the other encoding a library of DNA fragments, and transforming them into *E. coli.*[19] If a library encoded peptide is present in the library that associates with the target peptide, a complex between the library encoded peptide and the target peptide is formed.[20] The complex blocks certain operator regions in *E. coli*, making it resistant to phage infection challenge.[21] Resistant colonies may then be selected, and the library encoded peptides that bind to the target peptide isolated.[22] As one example, Kodadek discloses utilizing as the target peptide a 13-residue sequence from the protease cleavage site of the human insulin-like growth factor I (IGF-I).[23]

In contrast to the Appellants' claimed invention, Kodadek is concerned with the selection of library encoded peptides having affinity to a single, specific, known target peptide, not a surface with a multitude of unknown epitopes such as the surface of a fungus. Kodadek is not contacting his library encoded peptides with anything other than a provided target peptide (e.g., the protease cleavage site of the human insulin-like growth factor I) to identify binding partners to that particular target.

In essence, Kodadek is describing a prokaryotic analog of a conventional two-hybrid system, a well-known method for the detection of peptide-peptide interactions and the identification of genes encoding interactive protein. The development of the two-hybrid system is generally attributed to Fields et al., with early efforts described in U.S. Patent No. 5,283,173 (filed Jan. 24, 1990; issued Feb. 1, 1994); U.S. Patent No. 5,468,614 (filed Feb. 1, 1994; issued Nov. 21, 1995); and U.S. Patent No. 5,667,973 (filed Jun. 7, 1995, issued Sept. 16, 1997). Like Kodadek, the common two-hybrid system typically involves the interaction of peptides in a peptide library with a target peptide. Generally speaking, however, the two-hybrid system is a much more indirect assay as compared to that of Gough et al. Other than the mere fact

---

[19] Kodadek, page 4, paragraph [0042].
[20] *Id.*; *see also* Figure 1.
[21] *Id.*
[22] *Id.*
[23] *Id.* at page 4, paragraph [0039].

that peptides in a peptide library happen to be binding to other peptides (e.g., peptide epitopes), there is no apparent reason why one of skill in the art would be motivated to substitute a library of peptides into the methods of Gough et al. based on Kodadek's disclosure, or any other reference that merely describes the interaction of known peptides with a peptide library for that matter.

Furthermore, Kodadek actually **teaches away** from the use of phage display methods to identify library encoded peptides. Specifically, Kodadek suggests the superiority of his elaborate genetic selection scheme, where other methods have failed. In so doing, Kodadek notes that both **random peptides** (used in the present invention) and antibodies (used in Gough et al.) are **inadequate** for his purpose. As such, Kodadek and Gough et al. represent mutually exclusive domains, and, therefore, any suggestion of substitution of the peptides of Kodadek into the methods of Gough et al. would not be feasible. Thus, one skilled in the art would not and could not be motivated to substitute the highly specialized method of Kodadek into the method of Gough et al. without violating the objective of Gough et al. and rendering it unsatisfactory for its intended purpose.[24]

The Office has asserted that, by describing the disadvantages in the use of antibodies, Kodadek provided the motivation to substitute peptides into the methods of Gough et al. The Office's assertions apparently rest upon Kodadek's disclosure that "advances in the construction of single chain antibody libraries [scFv's] on phage promise to speed up this process."[25] However, in light of the fact that Kodadek discloses various drawbacks to the use of antibodies in binding studies, the quoted language is nothing more than a suggestion that the disadvantages of antibody usage may be minimized by phage display methodology.

More importantly, Kodadek, in addition to describing the disadvantages of antibodies, also discloses similar disadvantages of peptides, stating that "[u]nfortunately, peptides, or peptide epitopes in proteins, are difficult targets for molecular recognition in aqueous solution,"[26] and that early efforts by Kodadek and

---

[24] *See supra*, note 15.
[25] Kodadek, page 1, paragraph [0009] (internal citations omitted).
[26] *Id.* at paragraph [0006].

co-workers to isolate small peptides using phage display methods "***failed completely***."[27]

Unlike the case for antibodies, Kodadek is devoid of any suggestion that the disadvantages of peptide usage disclosed therein are minimized by any means. Instead, after encountering first-hand the limitation and/or failure of antibodies and phage display, Kodadek resorted to his genetic selection scheme described above to identify certain library encoded peptides having affinity to a given target peptide. Kodadek further describes that, in some cases, even the genetically selected library encoded peptides may not possess the desired affinity for the target.[28] In this event, Kodadek describes that conventional phage display methods may be modified by attaching the genetically selected library encoded peptide to the end of a standard phage displayed peptide library to form a "pincer."[29] The affinity of the genetically selected library encoded peptide arm is improved by the addition of the phage displayed peptide library because the library encoded peptide arm and the phage displayed random peptide library arm wrap around the target peptide.[30] Kodadek's specialized "pincer" approach thus facilitates identification of peptide-target interaction where the original peptide-epitope interaction (i.e., the library encoded peptide-known target interaction in the genetic selection scheme) is insufficient for stable interaction.[31]

Instead of providing motivation, a close reading of Kodadek clearly indicates that one skilled in the art would actually be guided away from combining the disclosures of Gough et al. and Kodadek. Keeping this standard in mind, it is significant that Kodadek refers to the futile attempts to identify peptide complexes using phage display that, in Kodadek's words, not only failed but "failed completely." Kodadek then goes on to describe his genetic selection scheme designed to be an improved method, in and of itself, to overcome past failures in identifying peptide complexes. It is only after particular, weakly-binding library encoded peptides that bind to the known target peptide

---

[27] Kodadek, page 4, paragraph [0038]. Appellants respectfully submit that it is the Office, not the Appellants, which has taken Kodadek's disclosure out of context on this point. (See Office action mailed July 25, 2005, at pages 6-7). Clearly Kodadek is referring to the failure of phage display as supporting the biases against the use of peptides.
[28] *Id.* at pages 13-14, paragraphs [0125]-[0134].
[29] *Id.* at page 14, paragraph [0132].
[30] *Id.* at paragraphs [0132]-[0133]; *see also* Kodadek, Figure 7.
[31] *Id.*

are identified by the genetic selection scheme (i.e., on the basis of affinity) that Kodadek coupled them with conventional phage display methods to form a pincer. Thus Kodadek clearly implies (and in some respects states outright) that conventional phage display methods, standing on their own, would not work. Why else would Kodadek go to all the trouble of devising the genetic selection scheme if random peptide phage display alone would be effective?

3. Petrenko et al.

Petrenko et al. describe methods of forming phage-displayed "landscape libraries" having complex surface functions that would be useful, e.g., in nanotechnology applications. According to Petrenko et al., the complex surface functions of phage clones depend on interactions between neighboring groups of display peptides and wild-type peptides.[32] The emergent properties of the phage surface inhere (i.e., are intrinsic) in the entire surface of the phage, not in the display peptides themselves.[33] Stated another way, Petrenko et al. describe modifications to phage such that the phage will display "global properties" across the entire surface of the phage, not only mere localized properties of the particular displayed peptides. As an example, Petrenko et al. suggest as desirable a phage with a high affinity for a metal ion that displays metal complexed on the surface in a specific repeating geometry.[34] Petrenko et al. also specifically disclose experiments where phage clones were selected for the "global property" of chloroform resistance.[35] Petrenko et al. also describe panning phage displayed peptides against a particular known target material. Such targets included dioxin in one experiment and the lectin concanavalin A in another experiment. In both cases, the phage displayed library of peptides was panned against a **single known target** (i.e., not a multitude of unknown targets such as those on the surface of a fungus).

The lack of teaching, suggestion, or motivation in Gough et al. and Kodadek is not remedied by the disclosure of Petrenko et al. Petrenko et al. describe panning phage displayed peptides against, like Kodadek, a **single known target** seeking to

---

[32] Petrenko et al. at page 797.
[33] *Id.*
[34] *Id.* at 801.
[35] *Id.* at 789-799.

identify phage clones that exhibit "global properties" across the entire phage surface (e.g., chloroform resistance), irrespective of the particular peptides of the library. The "global functions" described in Petrenko et al. were a means to select phage clones that were sensitive to chloroform and to demonstrate that chloroform resistance of the phage depended on the global surface properties of the phage. For example, in Petrenko et al., the mosaic phage was up to 10,000 times more sensitive to chloroform than the corresponding non-mosaic phage.[36]

This is in sharp contrast to the methods of Gough et al., where the target was a multitude of unknown surface epitopes presented on the surface of *Phytophthora*, and the authors were seeking to identify specific antibodies that bind to the surface of *Phytophthora*. Therefore, one skilled in the art would not and could not be motivated to modify the methods of Gough et al. according to the methods described in Petrenko et al. as such a modification would fail to achieve the clearly-stated objectives of each reference.[37]

Moreover, at no point in Petrenko et al. and/or Gough et al. is there any discussion, disclosure, or inference about how the localized properties of phage-peptide interactions provide an advantage over phage-antibody fragments for the isolation of antibodies. It is not enough that the peptide libraries, or specifically Petrenko et al.'s f8-1 peptide library, could be theoretically substituted into the methods of Gough et al.[38]

In contrast to the focus and methods of Petrenko et al., claim 1 is directed to the identification of peptides displayed on the surface of a vector that have a binding affinity for epitopes displayed on the surface of *Phytophthora*. This goal does not necessarily implicate "global functions" that inhere in the entire surface of phage-peptide. In fact, claim 1 does not even require that the peptide library be expressed on phage. Rather, the peptide library is expressed on a vector, and any vector capable of expressing the peptides of the peptide library may be used.[39] Contrary to the Office's assertion otherwise, the mosaic nature of phage-displayed peptides in Petrenko et al. provides

---

[36] Petrenko et al. at page 799.
[37] *See supra,* note 15.
[38] In re Mills, 916 F.2d 680, 16 USPQ2d 1430 (Fed. Cir. 1990); M.P.E.P. §2143.01. The mere fact that references can be combined or modified does not render the resultant combination obvious unless the prior art also suggests the desirability of the combination.
[39] See Appellants' Specification, paragraph [0049].

neither suggestion nor motivation to substitute peptides for antibody fragments in the method of Gough et al. in order to identify non-immunoglobulin peptides with affinity for surface epitopes of *Phytophthora*.

Finally, the Office asserts that Petrenko et al. extol the "global functions" that inhere in the entire surface landscape of phage-peptide as motivation to substitute peptides for antibody fragments in the method of Gough et al. As stated above, however, the objectives of Petrenko et al. (global functions) and Gough et al. (antibodies) are far too disparate for one skilled in the art to make such a substitution, particularly since such a substitution would render the prior art being modified unsatisfactory for its intended purpose.[40]

4. The Prior Art Provides No Reasonable Expectation of Success

Collectively, and individually, Gough et al., Kodadek and Petrenko et al. fail to disclose a method for the identification of a peptide, immunoglobulin or otherwise, having anti-fungal properties. Gough et al. merely identified immunoglobulin peptides that bind to the surface of *Phytophthora*; Gough et al. **failed** to demonstrate that their scFvs had any antifungal effect and merely expressed some vague hope that **scFvs** may ***one day*** "provide new tools to probe and manipulate pathogenicity."[41] Kodadek was not concerned with the identification of peptides having antifungal properties. Instead, Kodadek was concerned with two-hybrid methods and modifications thereto for isolating small peptides that recognize specific, known target peptides for use in affinity purification; significantly, however, Kodadek developed this approach because **the random peptide approach** (used in the present invention) and antibodies (used in Gough et al.) **were inadequate** for his purpose. Petrenko et al. were concerned with forming phage-displayed "landscape libraries" of general applicability and did not suggest any means for identifying antifungal peptides.

Against this backdrop, claim 1 defines a method which has been successfully used to identify non-immunoglobulin peptides which has been demonstrated to yield peptides having antifungal properties. According to the Office, this approach was obvious despite the fact that Gough et al., the only reference cited by the Office relating

---

[40] *See supra,* note 15.
[41] Gough et al. at page 107.

to antifungal peptides, **failed to identify any peptides having antifungal properties** and Kodadek said the random peptide approach **failed completely.**[42] Somehow, the Office has concluded a person of ordinary skill would have been led to adopt the method of claim 1 with an expectation of success despite the fact that not one of the three references cited by the Office successfully accomplished this and one of them said that prior attempts to use applicants' approach failed completely.[43] Simply stated, the Office's rejection of claim 1 is nothing more and nothing less than an impermissible hindsight rejection, using Appellants' disclosure as a template.[44]

B. The Group II Claims

Claim 9 is representative of the Group II claims. It depends from claim 1 (or claim 48) and additionally requires that each of the peptides be the same length, the length being 6 to 15 amino acids.[45]

1. Gough et al.

As discussed above, Gough et al. describe methods for the isolation of antibodies specific for surface-exposed epitopes on certain species of *Phytophthora* to be used for production of immunological probes and single-chain Fv (scFv) antibodies.[46] In contrast to the methods of Gough et al., claim 9 requires the use of peptides that are the same length, the length being 6 to 15 amino acids, not scFv antibody fragments.

The single-chain Fv antibodies utilized in the methods of Gough et al. are far more complex than the simple, 6- to 15-mer random peptides required by claim 9. In general, Gough et al.'s scFv antibody fragments may have a molecular weight of around

---

[42] Kodadek, page 4, paragraph [0038].

[43] Amgen, Inc. v. Chugai Pharmaceutical Co., 927 F.2d 1200, 1207-8, 18 USPQ2d 1016, 1022-23 (Fed. Cir. 1991) *cert. denied*, 502 U.S. 856 (1991).

[44] M.P.E.P. §2141; U.S. v. Adams, 383 U.S. 39 (1965); Panduit Corp. v. Dennison Mfg. Co., 774 F.2d 1082, 227 USPQ 337 (Fed. Cir. 1985), *vacated and remanded on other grounds*, 475 U.S. 809, 106 S.Ct. 1578 (1986), *adhered to on remand*, 810 F.2d 1561, 1 USPQ2d 1593 (Fed. Cir. 1987); W.L. Gore & Assoc., Inc. v. Garlock, Inc., 721 F.2d 1540, 220 U.S.P.Q. 303, 313 (Fed. Cir. 1983), *cert. denied*, 469 U.S. 851 (1984).

[45] The Group II claims are claims 9 and 41-44. Claims 41 and 43 are comparable to claim 9 except that they require the peptides to be 8 amino acids and 15 amino acids in length, respectively. Claims 42 and 44 require the peptide library to be the f8-1 and the f88-4 peptide library, respectively. As described in Appellants' Specification at paragraph [0049], the f8-1 peptide library includes random peptides that are 8 amino acids in length and the f88-4 peptide library includes random peptides that are 15 amino acids in length.

[46] *See supra* Section VIII(A)(1).

29,000.[47] Based on a molecular weight of 29,000 and using an average molecular weight of 137 for the 20 possible amino acids, Gough et al.'s antibody fragments would be about 212 amino acids in length; thus, Gough et al.'s antibody fragments are, at a minimum, 14 times longer than the 6- to 15-mer peptides required by claim 9. In addition, Gough et al.'s single-chain Fv antibodies include ancillary protein sequences that are involved in antibody structure and form, and may not work outside or independent of the complex phage-antibody framework.[48] Thus, not only do Gough et al. fail to teach or suggest the use of peptides in their methods, they also fail to teach or suggest peptides having the same length, the length being 6 to 15 amino acids.

A person of skill in the art must ignore the express teachings of Gough et al. to arrive at peptides having the same length, the length being 6 to 15 amino acids. Most significantly, Gough et al.'s approach "showed no detectable antifungal activity for any of the antibodies," and, at best, they hold out a vague hope for the future limited only to scFv antibodies and not peptides of a specific length.[49]

2. Kodadek

As discussed in detail above, Kodadek is concerned with the selection of library encoded peptides having an affinity to a single, specific, known target peptide, not a surface with a multitude of unknown epitopes such as the surface of a fungus. To accomplish his goals, Kodadek describes modifications to a conventional two-hybrid system. In so doing, Kodadek notes that both **random peptides** (e.g., 6 to 15-mer peptides required by claim 9) and antibodies (used in Gough et al.) are **inadequate** for his purpose. Most significantly, at the time of the present invention Kodadek had already stated that phage display methods were not only ineffective in identifying small peptides, they were a ***complete failure***.[50] Thus, Kodadek **teaches away** from the use of phage display methods to identify library encoded peptides, regardless of length.

3. Petrenko et al.

Petrenko et al., like Kodadek, describe panning phage-displayed peptides against a single known target seeking to identify phage clones that exhibit "global

---

[47] *See, e.g.*, Glockshuber et al., A comparison of strategies to stabilize immunoglobulin Fv-fragments, Biochemistry (1990), 29(6):1362-7.
[48] *See supra* Section VIII(A)(1).
[49] *Id. See also* Gough et al. at page 107.
[50] *See supra* Section VIII(A)(2). *See also* Kodadek, page 4, paragraph [0038].

properties" across the entire phage surface. That is, Petrenko et al. describe modifications to phage such that the phage will display "global properties" across the entire surface of phage, not only mere localized properties of the particular displayed peptides.[51] This is in sharp contrast to the methods of Gough et al., where the target was a multitude of unknown surface epitopes presented on the surface of *Phytophthora* and the authors were seeking to identify specific antibodies that bind to the surface of *Phytophthora*.

4. The Prior Art Provides No Reasonable Expectation of Success

At the time of Appellants' invention, Petrenko et al.'s peptide libraries were known in the art, and Gough et al. still selected a phage-antibody library of scFv fragments. Incredibly, the Office has nonetheless concluded that Petrenko et al.'s phage-displayed peptides could be substituted into the methods of Gough et al. The Office makes this assertion despite the fact that Gough et al.'s method "showed no detectable antifungal activity for any of the antibodies" and Kodadek said it would not work. There is simply ***no reason*** to believe that the substitution of smaller peptides such as those having a length of 6 to 15 amino acids would even work, let alone provide an improvement over the larger scFv antibodies of Gough et al.

IX. CONCLUSION

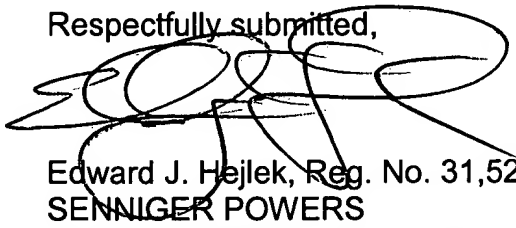
A *prima facie* case of obviousness has not been established pursuant to 35 U.S.C. § 103(a) based on the combined references of Gough et al., Kodadek, and Petrenko et al. It has not been shown that the cited references would have motivated a person of ordinary skill in the art to make the Appellants' invention, would have provided a reasonable expectation of success, or when considered as a whole, would have suggested all of the requirements of the claimed invention. For these reasons, and for those more fully stated above, Appellants respectfully request the rejections be reversed and claims 1-9 and 32-51 be allowed.

A check and fee transmittal in the amount of $760.00 are enclosed for payment of the fees related to this filing ($510.00 for a three-month extension of time; and

[51] *See supra* Section VIII(A)(3). *See also* Petrenko et al. at page 797.

$250.00 for the Appeal Brief). The Commissioner is hereby authorized to charge any additional fees which may be required to Deposit Account No. 19-1345.

Respectfully submitted,



Edward J. Hejlek, Reg. No. 31,525
SENNIGER POWERS
One Metropolitan Square, 16th Floor
St. Louis, Missouri 63102
(314) 231-5400

EJH/BJS/clh

Express Mail Label No. EV 775628500 US
Mail Stop AF

CLAIMS APPENDIX

1. (previously presented) A method for identification of non-immunoglobulin peptides having an affinity for the surface of a fungus comprising:

(a) constructing a library of peptides by,

(i) preparing random oligonucleotides;

(ii) inserting said oligonucleotides into a vector that expresses peptides encoded by said random oligonucleotides on its surface and is capable of transfecting a host cell;

(iii) transfecting a host cell with said vector to amplify said vector in an infectious form to create a library of peptides on the surface of said vector;

(b) contacting said vector expressing said peptide library with a target fungus and removing unbound vector;

(c) eluting bound vector from said fungus;

(d) amplifying said bound vector;

(e) sequencing the oligonucleotides contained in said eluted vector;

(f) deducing the amino acid sequence of peptides encoded by said oligonucleotides contained in said eluted vector; and

(g) selecting the non-immunoglobulin peptides for which the amino acid sequence has been deduced.

2. (previously presented) The method of any one of claims 1, 48, or 49 further comprising repeating steps (b) through (d) at least once.

3. (previously presented) The method of any one of claims 1, 48, or 49, wherein said vector is a fusion phage vector.

4. (previously presented) The method of any one of claims 1, 48, or 49, wherein said vector is a fusion phage vector selected from the group consisting of type 8,

type 88, type 8+8, type 3, type 33, type 3+3, type 6, type 66, type 6+6, phage T7 and phage 8.

5. (previously presented) The method of any one of claims 1 or 48, wherein the sequence of said random oligonucleotide is GCA GNN (NNN)7 or SEQ ID NO: 1.

6. (previously presented) The method of any one of claims 1, 48, or 49, wherein said peptide is expressed as part of a coat protein of said vector.

7. (original) The method of claim 6, wherein said coat protein is a pIII or a pVIII coat protein.

8. (previously presented) The method of any one of claims 1, 48, or 49, further comprising determining the binding affinity of said peptides to said target fungus.

9. (previously presented) The method of any one of claims 1 or 48, wherein each of said peptides are of the same length, the length being 6 to 15 amino acids.

10-31. (canceled)

32. (previously presented) The method of any one of claims 1 or 49 wherein the target fungus is a plant pathogenic fungus.

33. (previously presented) The method of any one of claims 1 or 49 wherein the target fungus is a member of genus *Phytophthora*.

34. (previously presented) The method of any one of claims 1 or 49 wherein the target fungus is selected from the group consisting of *Phytophthora sojae*, *Phytophthora capsici*, *Phytophthora cactorum*, *Phytophthora palmivora*, *Phytophthora cinnamomi*, *Phytophthora infestans*, and *Phytophthora parasitica*.

35. (previously presented) The method of any one of claims 1 or 49 wherein the target fungus is selected from the group consisting of *Phytophthora sojae*, *Phytophthora capsici*, *Phytophthora palmivora*, *Phytophthora cinnamomi*, and *Phytophthora parasitica*.

36. (previously presented) The method of any one of claims 1 or 49 wherein the target fungus is *Phytophthora sojae* or *Phytophthora capsici*.

37. (previously presented) The method of any one of claims 1, 48, or 49 wherein the vector expressing the peptide library is contacted with the target fungus at different life stages of the target fungus.

38. (previously presented) The method of any one of claims 1, 48, or 49 wherein the vector expressing the peptide library is contacted with the target fungus at oospore life stage or chlamydospore life stage.

39. (previously presented) The method of any one of claims 1, 48, or 49 wherein the vector expressing the peptide library is contacted with the target fungus at zoospore life stage.

40. (previously presented) The method of any one of claims 1, 48, or 49 wherein the vector expressing the peptide library is contacted with the target fungus at germling life stage.

41. (previously presented) The method of any one of claims 1 or 48 wherein each of said peptides are of a same length, the length being 8 amino acids.

42. (previously presented) The method of any one of claims 1 or 48 wherein the peptide library is an f8-1 peptide library.

43. (previously presented) The method of any one of claims 1 or 48 wherein each of said peptides are of a same length, the length being 15 amino acids.

44. (previously presented) The method of any one of claims 1 or 48 wherein the peptide library is an f88-4 peptide library.

45. (previously presented) The method of any one of claims 1, 48, or 49, further comprising repeating steps (b) through (d) at least twice.

46. (previously presented) The method of any one of claims 1, 48, or 49, further comprising repeating steps (b) through (d) at least three times.

47. (previously presented) The method of any one of claims 1, 48, or 49 wherein the bound vector is amplified in an *E. coli*.

48. (previously presented) A method for identification of non-immunoglobulin peptides having an affinity for the surface of a fungus comprising:

(a) constructing a library of peptides by,

(i) preparing random oligonucleotides;

(ii) inserting said oligonucleotides into a vector that expresses peptides encoded by said random oligonucleotides on its surface and is capable of transfecting a host cell;

(iii) transfecting a host cell with said vector to amplify said vector in an infectious form to create a library of peptides on the surface of said vector;

(b) contacting said vector expressing said peptide library with a target fungus and removing unbound vector, wherein the target fungus is selected from the group consisting of *Phytophthora sojae*, *Phytophthora capsici*, *Phytophthora palmivora*, *Phytophthora cinnamomi*, and *Phytophthora parasitica*;

(c) eluting bound vector from said fungus;

(d) amplifying said bound vector;

(e) sequencing the oligonucleotides contained in said eluted vector;

(f) deducing the amino acid sequence of peptides encoded by said oligonucleotides contained in said eluted vector; and

(g) selecting the non-immunoglobulin peptides for which the amino acid sequence has been deduced.

49. (previously presented) A method for identification of non-immunoglobulin peptides having an affinity for the surface of a fungus comprising:

(a) constructing a library of peptides by,

(i) preparing random oligonucleotides;

(ii) inserting said oligonucleotides into a vector that expresses peptides encoded by said random oligonucleotides on its surface and is capable of transfecting a host cell;

(iii) transfecting a host cell with said vector to amplify said vector in an infectious form to create a library of peptides on the surface of said vector;

wherein the library of peptides is (1) an f8-1 peptide library, wherein each peptide of the f8-1 peptide library has a length of 8 amino acids or (2) an f88-4 peptide library, wherein each peptide of the f88-4 peptide library has a length of 15 amino acids;

(b) contacting said vector expressing said peptide library with a target fungus and removing unbound vector;

(c) eluting bound vector from said fungus;

(d) amplifying said bound vector;

(e) sequencing the oligonucleotides contained in said eluted vector;

(f) deducing the amino acid sequence of peptides encoded by said oligonucleotides contained in said eluted vector; and

(g) selecting the non-immunoglobulin peptides for which the amino acid sequence has been deduced.

50. (previously presented) The method of claim 49 wherein the library of peptides is a f8-1 peptide library, wherein each peptide of the f8-1 peptide library has a length of 8 amino acids.

51. (previously presented) The method of claim 49 wherein the library of peptides is a f88-4 peptide library, wherein each peptide of the f88-4 peptide library has a length of 15 amino acids.

EVIDENCE APPENDIX

Appellants rely on the Glockshuber et al. reference (Biochemistry (1990), 29(6): 1362-7) and the Fields et al. references (U.S. Patent Nos. 5,283,173; 5,468,614; and 5,667,973) to support the above arguments. Appellants enclose herewith copies of the Glockshuber et al. and Fields et al. references.

RELATED PROCEEDINGS APPENDIX

Not applicable.

A Comparison of Strategies To Stabilize Immunoglobulin F_v-Fragments†

Rudi Glockshuber, Mark Malia, Ilse Pfitzinger, and Andreas Plückthun*
Genzentrum der Universität München, Max-Planck-Institut für Biochemie, Am Klopferspitz, D-8033 Martinsried, FRG
Received November 7, 1989

ABSTRACT: F_v-Fragments of antibodies may dissociate at low protein concentrations and are too unstable for many applications at physiological temperatures. To stabilize F_v-fragments against dissociation, we have tested and compared three different strategies on the F_v-fragment of the well-characterized phosphocholine binding antibody McPC603 expressed and secreted in *Escherichia coli*: chemical cross-linking of the variable domains, introduction of an intermolecular disulfide bond, and construction of a peptide linker to produce a "single-chain" F_v-fragment. All the linked fragments show hapten affinities nearly identical with that of the whole antibody independent of protein concentration and are significantly (up to 60-fold) stabilized against irreversible thermal denaturation. All genetically engineered linked F_v-fragments can be obtained in native conformation in *E. coli*. The reported strategies for generating F_v-fragments with improved physicochemical properties may extend their usefulness in biotechnology as well as in therapeutic and diagnostic applications.

Immunoglobulin F_v-fragments (heterodimers consisting of only the variable domains V_H and V_L) are probably the minimal fragments of an antibody required for antigen binding activity. Their small size makes them interesting targets in the development of immunodiagnostic and immunotherapeutic applications since they may be expected to have better properties for penetration of solid tumor tissue as well as lower antigenicity and improved pharmacokinetics (Sedlacek et al., 1988). Furthermore, they are ideal models for protein engineering studies on antibodies as they provide the opportunity to investigate the binding properties of antibodies with a very small protein that is potentially amenable to structural analysis by NMR and crystallography. However, we have now found that F_v-fragments show a surprisingly limited stability at low protein concentration and under physiological conditions. This fact prompted us to investigate ways of improving their stability.

The studies described here were carried out with the F_v-fragment of the well-characterized phosphocholine binding IgA McPC603, for which the three-dimensional structure is known (Satow et al., 1986; Segal et al., 1974). We have previously developed a very convenient system for the expression of fully functional F_v-fragments from *Escherichia coli* (Skerra & Plückthun, 1988; Plückthun et al., 1987) and have shown that the recombinant F_v-fragment expressed in *E. coli* has nearly the same binding constant for the hapten as does the whole antibody (Skerra & Plückthun, 1988; see below).

Protein engineering on immunoglobulins may become an important part in the development of improved antibodies for diagnostics, therapy, and industrial applications such as protein purification or catalysis [reviewed, e.g., in Kraut (1988), Schultz (1988), Lerner and Bencovic (1988), and Plückthun et al. (1987)]. Rational approaches to the alteration of the binding properties of any protein are still at an early stage. Their development requires the availability of well-characterized model systems for antibody combining sites. With small, well-studied antigen binding fragments directly expressed in bacteria, random mutagenesis approaches may also be more easily carried out. All of these potential applications require a full understanding of the stability properties of various recombinant antigen binding fragments. Furthermore, their antigen binding properties must be critically compared with the native antibody to evaluate the suitability of various recombinant fragments as model systems for the binding properties of the whole antibody. These questions are addressed for the F_v-fragment of the antibody McPC603 in this paper.

Experimental Procedures

Recombinant DNA Techniques and Protein Expression. Recombinant DNA techniques were based on Maniatis et al. (1982). The antibody fragments were expressed in *E. coli* JM83 (Vieira & Messing, 1982) with a vector similar to that described (Skerra & Plückthun, 1988) but containing an M13 phage origin (Vieira & Messing, 1987; Skerra and Plückthun, unpublished results). Site-directed mutagenesis was carried out according to Kunkel et al. (1987).

Protein Purification. The recombinant antibody fragments were purified by phosphocholine affinity chromatography essentially as described previously (Skerra & Plückthun, 1988) except that the bacterial growth was performed at 20 °C and the cells were induced for 3 h before the harvest. The cells were then disrupted in a French pressure cell, and the soluble part of the lysate was directly applied onto the affinity column.

The F_{ab}'-fragment of McPC603 was prepared essentially as described (Rudikoff et al., 1972).

Cross-Linking. The cross-linking with glutaraldehyde (0.10 M) was carried out in BBS buffer (0.16 M NaCl, 0.20 M borate/NaOH, pH 8.0) for 1 min at 20 °C. After addition of $NaBH_4$ to a final concentration of 0.10 M and incubation for 20 min (20 °C), the samples were dialyzed against BBS.

The glutaraldehyde-cross-linked F_v-fragment was obtained on a preparative scale by cross-linking under the same conditions as in the analytical experiments at a protein concentration of 2 μM in the presence of 5 mM phosphocholine. The hapten was then removed by dialysis, and the cross-linked material was purified by affinity chromatography.

Hapten Binding. (*a*) *Fluorescence Measurements.* The fluorescence measurements were performed in BBS at 20 °C. The tyrosine and tryptophan fluorescence (excitation at 280 nm) was recorded for 5 s at 328 nm and averaged for each titration point. For the Scatchard analysis, the fluorescence

†Supported by Grant BCT0372 from the Bundesministerium für Forschung und Technologie to A.P.
*To whom correspondence should be addressed.

0006-2960/90/0429-1362$02.50/0

change was recorded as a function of PC addition. The fraction of protein with bound hapten r was determined as $(F - F_0)/(F_{max} - F_0)$, where F is the measured fluorescence, F_0 the fluorescence without hapten, and F_{max} the fluorescence in the presence of 10 mM PC. The concentration of free PC was calculated from the known protein concentration, the total PC concentration, and r. The protein molarity was determined by OD_{205}, assuming (Scopes, 1982) an extinction coefficient of $\epsilon^{0.1\%} = 31.0$.

(*b*) *Equilibrium Dialysis.* The equilibrium dialysis measurements were carried out as described previously (Skerra & Plückthun, 1988).

Protein Stability. The F_v-fragments were incubated at identical protein concentrations (1.30 μM) in BBS at 37 °C. After different times of incubation (0–24 h), samples of 100 μL were removed and centrifuged. Each supernatant (75 μL) was applied onto a 14% SDS-PAGE (Fling & Gregerson, 1986), and the amount of soluble protein was determined densitometrically with bovine serum albumin (BSA) as an internal standard (see Figure 1c). The first-order kinetics obtained had correlation coefficients of 0.99 and were used to obtain the half-lives in Table I.

Other Methods. The relative functional expression of the various fragments was determined as the approximate relative amounts purified by affinity chromatography from cells grown under identical conditions.

Results and Discussion

Preliminary results had suggested that the F_v-fragment of the antibody McPC603 might dissociate at high dilution (Plückthun et al., 1988, 1989). We have now examined this finding in detail by covalently cross-linking (Jaenicke & Rudolph, 1986) the variable domains of the F_v-fragment with glutaraldehyde at different protein concentrations. From the observed concentration-dependent dissociation equilibrium, we deduce that the equilibrium constant for the dissociation of the two domains of the F_v-fragment is on the order of 10^{-6} M (Figure 1a).

To investigate the effect of the dissociation of the F_v-fragment on its apparent binding properties, we have analyzed its hapten affinity in more detail and compared it with that of the proteolytically prepared F_{ab}'-fragment of McPC603. This was achieved by fluorescence titration experiments, which are made possible by an increase in protein fluorescence induced by hapten binding (Glaudemans et al., 1977; Pollet & Edelhoch, 1973). The hapten-induced fluorescence change can only be observed when the excitation is carried out at 280 nm; at 295 nm there is essentially no observed fluorescence increase. This suggests the involvement of tyrosine residues in the process causing the fluorescence change.

The affinity constant of the F_{ab}'-fragment for phosphocholine of 1.6×10^5 M^{-1} so obtained was found to be identical with the value reported for the whole antibody (Metzger et al., 1971). In contrast, the dissociation of the F_v-fragment led to distinctly lower *apparent* hapten binding constants of the F_v-fragment as measured by fluorescence, which were found to be dependent on protein concentration (Figure 2a). The curved appearance of the Scatchard plot is caused by the fact that the fluorescence measurement also records changes in domain association, which in turn depends on hapten binding. These results are entirely consistent with our previously reported affinity constant for the F_v-fragment (Skerra & Plückthun, 1988) of 1.2×10^5 M^{-1}, which had been determined by equilibrium dialysis at high protein concentration (8.5 μM). This measured value is reproduced by calculation, assuming an intrinsic hapten binding constant identical with that of the

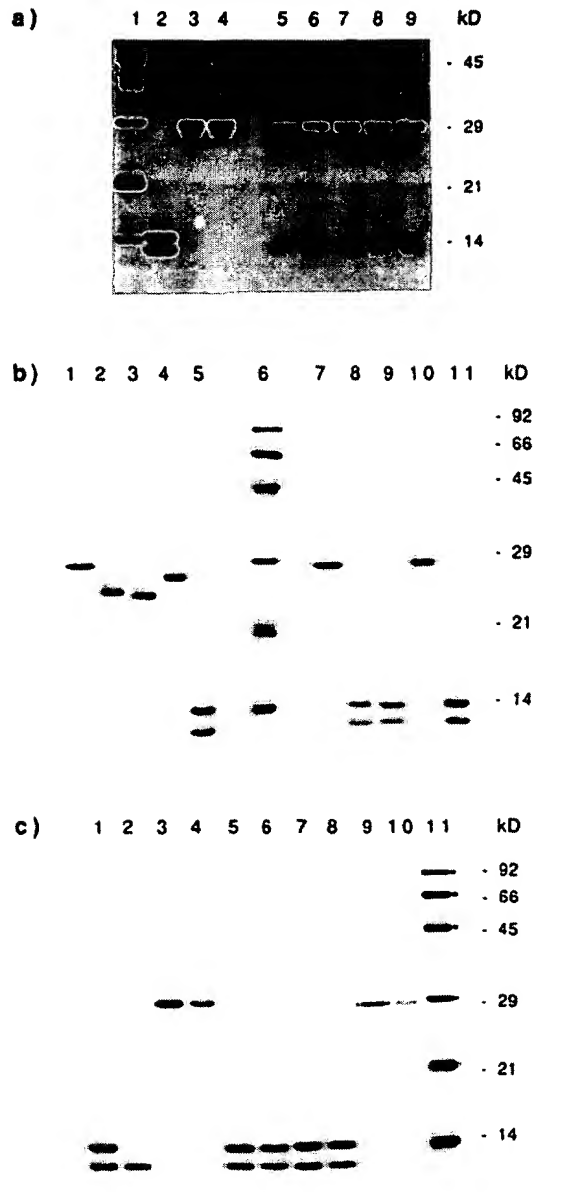


FIGURE 1: (a) Covalent cross-linking of the native F_v-fragment with glutaraldehyde at different protein concentrations. A silver-stained SDS-PAGE (14%) is shown: (lane 1) protein molecular size marker; (lane 2) native F_v-fragment; (lanes 3 and 4) F_v-fragment, cross-linked in the presence of 5 mM phosphocholine at protein concentrations of 0.8 and 12.0 μM, respectively; (lanes 5–9) F_v-fragment, cross-linked at protein concentrations of 0.8, 1.5, 3.0, 6.0, and 12.0 μM, respectively, but in the absence of the hapten. The cross-linking with glutaraldehyde was carried out as described under Experimental Procedures. (b) Purification of the native F_v-fragment and of the various covalently linked F_v-fragments. An SDS-PAGE (14%) stained with Coomassie brilliant blue is shown. The samples in lanes 6–11 were reduced by boiling with β-mercaptoethanol, and the samples in lanes 1–5 were not reduced. (Lanes 1 and 7) Single-chain F_v-fragment; (lanes 2 and 8) disulfide-linked mutant 56–106; (lanes 3 and 9) disulfide-linked mutant 55–108; (lanes 4 and 10) chemically cross-linked F_v-fragment; (lanes 5 and 11) native F_v-fragment; (lane 6) protein molecular size marker. (c) Thermal stability of the native and the cross-linked F_v-fragments. An SDS-PAGE (14%) stained with Coomassie brilliant blue is shown. (Lanes 1 and 2) Native F_v-fragment; (lanes 3 and 4) chemically cross-linked F_v-fragment; (lanes 5 and 6) mutant 55–108; (lanes 7 and 8) mutant 56–108; (lanes 9 and 10) single-chain F_v-fragment; (lane 11) protein molecular size marker. Identical amounts of the soluble fraction of the protein solutions before (corresponding left lanes) and after (right lanes) a 24-h incubation at 37 °C were applied to the gel. The internal standards were omitted for the gel shown here as an example of a 24-h incubation.

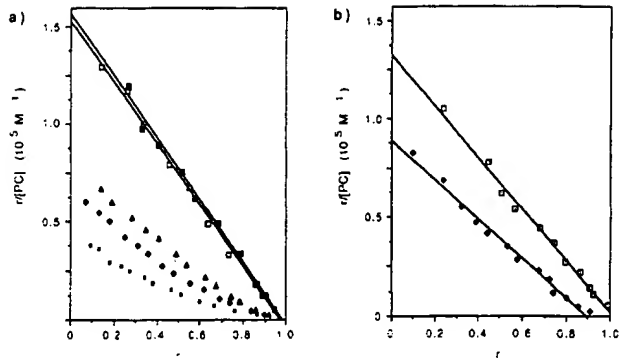


FIGURE 2: Fluorescence changes upon binding phosphocholine (PC). (a) Scatchard plot of the binding of PC to the native F_v-fragment at various protein concentrations [(△) 2.89, (◇) 0.96, and (small solid squares) 0.32 μM] compared with the proteolytically prepared F_{ab}'-fragment [(large solid squares) 0.96 μM] and the chemically crosslinked F_v-fragment [(□) 0.96 μM]. (b) Scatchard plot of PC binding to the genetically engineered cross-linked F_v-fragments: (⊡) single-chain F_v-fragment; (◆) 55–108. The fluorescence measurements were performed as described under Experimental Procedures. *r* denotes the fraction of antibody fragment with bound hapten, and [PC] denotes the concentration of free phosphocholine.

Table I: Properties of Covalently Linked F_v-Fragments

fragment[a]	K_{assoc}^{PC} (10^5 M^{-1}) fluor	K_{assoc}^{PC} (10^5 M^{-1}) eq dial	relative functional expression in vivo	half-life of denaturation at 37 °C (h)
native F_v (wt)	*b*	1.2[c]	1.0	1.3[d]
cl F_v	1.6	nd		25
55–108	1.0	1.0	0.2	88
56–106	nd[e]	0.7	0.2	70
sc F_v	1.3	nd	0.5	15

[a] The fragments are the unmodified F_v-fragment (denoted native F_v), the glutaraldehyde-cross-linked F_v (cl F_v), the disulfide-linked fragments L55–H108 (55–108) and L56–H106 (56–106), and the single-chain F_v-fragment (sc F_v). [b] Dependent on protein concentration because of the dissociation at low protein concentration (see text). [c] Measured at 8.5 μM (Skerra & Plückthun, 1988). [d] Denaturation of V_H. [e] No fluorescence change; see text.

F_{ab}'-fragment and the measured V_H:V_L dissociation constant for the F_v-fragment of 1×10^{-6} M.

We also observed a large difference between the thermal stability of the F_v-fragment and the F_{ab}'-fragment. While the F_{ab}'-fragment is fully stable at 37 °C for extended periods (data not shown), the V_H chain of the F_v-fragment rapidly and irreversibly denatures and precipitates (Figure 1c and Table I). This low stability makes the F_v-fragment unsuitable for many technical or medical applications. While the denaturation of V_H is rapid, the denaturation of the V_L chain is much slower. The different stabilities of the variable domains and the different stabilities of the F_v- and F_{ab}'-fragments are consistent with a pathway of protein denaturation in which the F_v-fragment dissociates first and then the domains unfold. These considerations led us to the hypothesis that the thermal stability of the F_v-fragment might be increased by a stronger association between V_L and V_H.

We therefore utilized three different strategies to stabilize the well-characterized F_v-fragment of the antibody McPC603 against dissociation by covalently linking the variable domains. Furthermore, in a covalently cross-linked fragment the hapten binding behavior should simply reflect the intrinsic binding properties of the whole antibody.

In the first approach, the F_v-fragment of McPC603 purified from *E. coli* was cross-linked with glutaraldehyde (Jaenicke & Rudolph, 1986) in the presence of phosphocholine. The cross-linked material was obtained with an overall yield of about 80% after purification to homogeneity (Figure 1b) with a phosphocholine affinity column (Chesebro & Metzger, 1972).

In a second approach, we constructed two different F_v-fragments each containing an intermolecular disulfide bond. To locate appropriate positions for cysteine residues, a collection of disulfide bonds from the protein data base was evaluated (Pabo & Suchanek, 1986). All possible amino acid pairs at the V_L:V_H interface whose main-chain atoms had RMS deviations of less than 2.0 Å from any member of this collection were identified. Of these candidate pairs, residues close to the hapten binding site and those involving proline residues were not considered. We report here the results on two molecules that were obtained by site-directed mutagenesis (Kunkel et al., 1987) [55–108, L55 Tyr → Cys, H108 Tyr → Cys; 56–106, L56 Gly → Cys, H106 Thr → Cys (Figure 3)]. We found that the intermolecular disulfide bond in both molecules had formed in the periplasm in vivo. In both mutant proteins, all the material isolated from a phosphocholine affinity column was covalently linked (Figure 1b), and all the covalently linked soluble material, as detected by Western blot from a nonreducing gel, binds to the affinity column (data not shown).

In the third approach, we constructed a secreted single-chain F_v-fragment. The intragenic region between V_H and V_L of the artificial operon (Skerra & Plückthun, 1988) as well as the signal sequence of the downstream gene encoding V_L was replaced by a DNA fragment encoding the sequence (Gly-Gly-Gly-Gly-Ser)$_3$ by site-directed mutagenesis. This F_v-fragment is thus encoded by a single peptide segment and contains one signal sequence. This signal is the same as that used for V_H in all other F_v-fragments reported here. This single-chain F_v-fragment was found to be secreted normally and could also be directly purified by affinity chromatography in a single step (Figure 1b). In contrast to the previously reported expression strategy of similar molecules (Bird et al., 1988; Huston et al., 1988), no in vitro refolding is necessary in our expression system.

The amounts of the various functional F_v-fragments obtained from *E. coli* were compared. None of the described strategies to improve the association of V_L and V_H resulted in higher yields of native cross-linked F_v-fragments than that obtained for the wild-type fragment with two unlinked chains (Table I). From this finding we deduce that it is *not* the association of the variable domains that limits the achievable expression level of functional F_v-fragments in *E. coli*. Rather, this result is consistent with an alternate view, that the amount of correctly folded F_v-fragment is determined by the folding process of the single domains both in vivo and in vitro (Hochman et al., 1976). The single-chain strategy therefore offers no particular advantage as far as the expression yield in *E. coli* is concerned. The single-chain fragment has advantages in stability (see below), however, and the secretion system described here provides an easier access to such linked fragments.

We then determined the hapten binding constants of all cross-linked F_v-fragments by fluorescence titration (Figure 2 and Table I). The affinities for phosphocholine were found to be essentially the same for the F_{ab}'-fragment and the chemically cross-linked F_v-fragment, which demonstrates that the intrinsic binding property of McPC603 is fully retained in its F_v-fragment. The hapten affinities of all other cross-linked species were only slightly reduced. Furthermore, the affinity constants of all cross-linked F_v-fragments were independent of protein concentration in contrast to those of the native F_v-fragment.

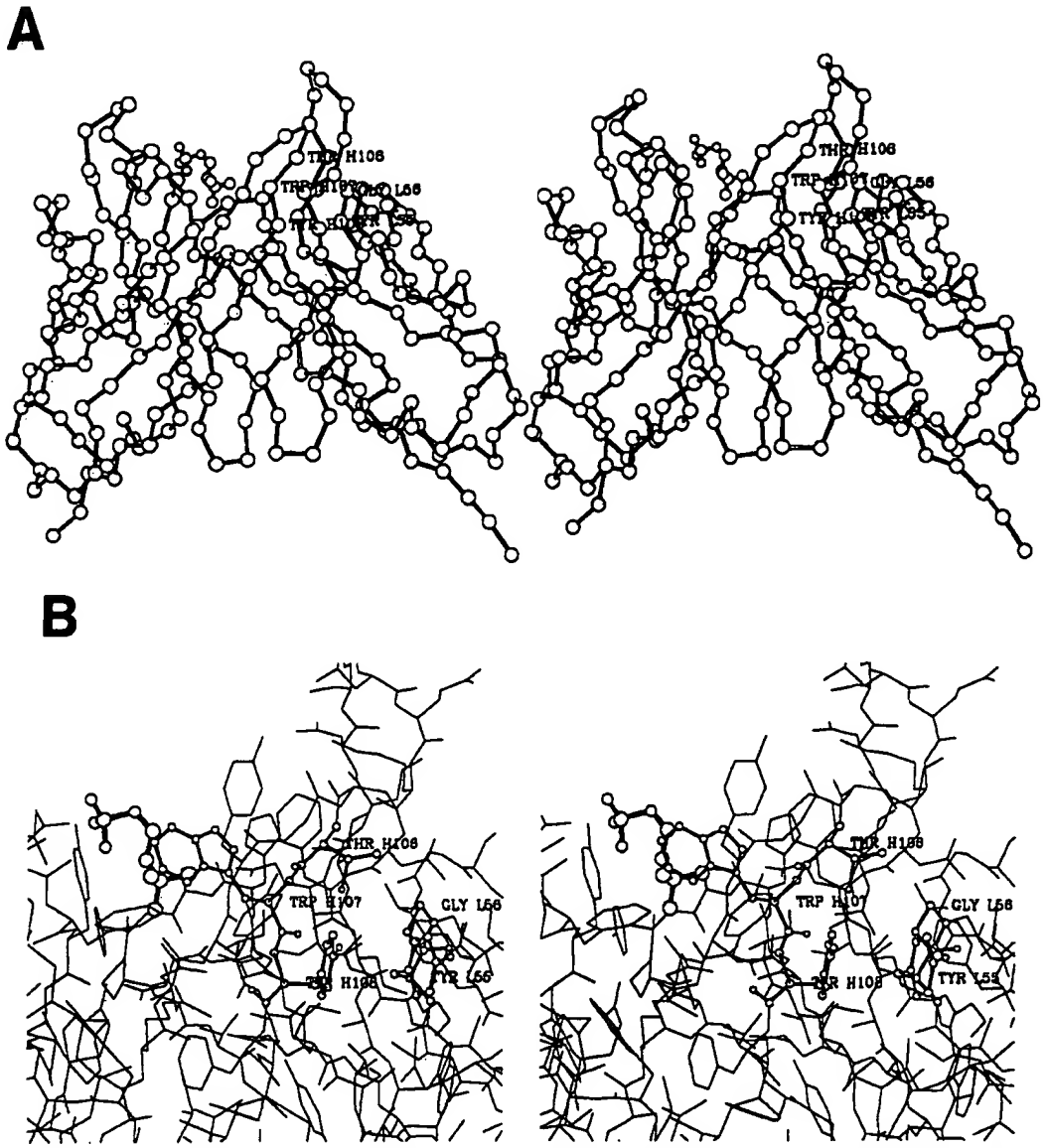


FIGURE 3: Position of the engineered intermolecular disulfide bonds. The pairs of residues Tyr L55 and Tyr H108 or Gly L56 and Thr H106 were changed to cysteines. The hapten phosphocholine is shown as a ball-and-stick model in both panels. In (A), only the α-carbon backbone is shown with the V_L domain having open bonds and V_H having filled bonds. The residue number is given on the right of the corresponding α-carbon atom. In (B), a close-up view of the region around the mutated residues is shown. The residues Tyr L55, Tyr H108, Gly L56, and Thr H106 (which were changed to cysteines) as well as Trp H107 are highlighted as ball-and-stick objects. The residue number is given on the right of the corresponding α-carbon atom. The coordinates are those of Davies and co-workers (Satow et al., 1986; Segal et al., 1974), and the plot program of Lesk and Hardman (1982) was used.

Surprisingly, no fluorescence change at all was observed for the mutant protein 56–106 upon addition of phosphocholine, although this protein bound to the phosphocholine affinity column and therefore could be purified to homogeneity. In contrast, mutant 55–108, in which two tyrosine residues were changed to cysteines, showed normal fluorescence changes. To clarify the behavior of these mutant proteins, their binding affinity to phosphocholine was determined by equilibrium dialysis. The value obtained for 56–106 (0.7×10^5 M^{-1}; Figure 4, Table I) shows that this mutant protein is able to bind phosphocholine with only a 2-fold reduction in hapten affinity compared with the native antibody. Both methods (fluorescence and equilibrium dialysis) gave identical values for the other disulfide mutant 55–108, validating both approaches. This protein was also found to bind the hapten with a similar affinity as the whole antibody. The reason for the unexpected lack of hapten-induced fluorescence change of the mutant protein 56–106 may be a tethering of the CDR-3 loop of V_H by the disulfide bond involving H106 (Figure 3). Tryptophan H107, which is directly in contact with the hapten, or another aromatic residue in the neighborhood, may be restricted in its movement by a disulfide bond at the tip of the loop (H106) but less so by a disulfide bond further away from the tip (H108) in the mutant 55–108.

Upon addition of the hapten to saturation, the change in tyrosine fluorescence of both the F_{ab}'-fragment and the chemically cross-linked F_v-fragment is a very fast process whose kinetics cannot be resolved without recourse to rapid-mixing techniques. In contrast, the natural F_v-fragment, the single-chain F_v-fragment, and the disulfide-linked F_v-fragment give rise to a fast step followed by a slow fluorescence change apparently displaying first-order kinetics on a time scale of minutes, similar to previously reported results (Watt & Voss, 1979). The rate of this slow process does not appear to depend on protein concentration. The physical basis of this slow fluorescence change needs to be further investigated. It might

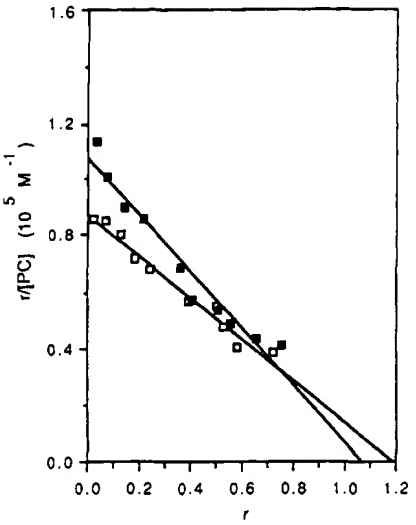


FIGURE 4: Scatchard plot of the equilibrium dialysis data for the binding of phospho[*methyl*-^{14}C]choline to the disulfide mutant proteins 55–108 and 56–106. *r* denotes the fraction of antibody fragment with bound hapten, and [PC] denotes the concentration of free phosphocholine. (■) Mutant protein 55–108 at 10 μM; (□) mutant protein 56–106 at 10 μM.

be reflected in the slight differences in apparent hapten binding constants and may also involve movement of the CDR3 loop in V_H.

The thermal stability of the various covalently linked F_v-fragments was measured by recording the decrease of soluble protein on incubation at 37 °C (Figure 1c). We found that all cross-linking strategies led to a strong stabilization of the corresponding proteins against irreversible denaturation. The data were analyzed according to first-order kinetics (Table I). The single-chain F_v-fragment, the least stable cross-linked protein, is still 10-fold more stable than the natural F_v-fragment. The disulfide-linked mutants 55–108 and 56–106 were found to be the most stable species with a 60- and 50-fold increase in the half-life at 37 °C, respectively. While V_H is much less stable than V_L in the natural F_v-fragment, the covalent linking improves the thermal stability of the linked fragments to at least that of V_L (Figure 1c).

We conclude from these results that the stability of the F_v-fragment of McPC603 is influenced by the interaction of the variable domains V_L and V_H. The association constants of antibody F_v-fragments that have been reported (Hochmann et al., 1976; Klein et al., 1979; Home et al., 1982) range from 10^8 to 10^5 M^{-1}, and there is probably no strong natural selection for the interactions of the variable domains of antibodies due to the simultaneous association of the constant regions of both chains. Since hapten binding and domain association are mutually dependent, the analysis of hapten binding at low concentrations of F_v-fragment is rather complex. We show here that, for detailed hapten binding studies involving site-directed mutagenesis, the chemically cross-linked F_v-fragment is especially suitable as its binding properties are essentially identical with those of the F_{ab}'-fragment, straightforward to analyze, and probably directly reflect the intrinsic hapten affinity constants. On the other hand, all three reported strategies for preventing the dissociation of V_L and V_H lead to F_v molecules with dramatically improved thermal stabilities with the disulfide-linked mutants giving the most pronounced improvement. The greatly increased lifetime at physiological temperatures of all covalently linked F_v-fragments could make such fragments very promising agents for medical and biotechnological applications.

ACKNOWLEDGMENTS

We thank A. Skerra for constructing the improved plasmid and Dr. C. Pabo (Johns Hopkins University) for the PROTEUS package of programs.

Registry No. Glutaraldehyde, 111-30-8.

REFERENCES

Bird, R. E., Hardman, K. D., Jacobson, J. W., Johnson, S., Kaufman, B. M., Lee, S. M., Lee, T., Pope, S. H., Riordan, G. S., & Whitlow, M. (1988) *Science 242*, 423–426.

Chesebro, B., & Metzger, H. (1972) *Biochemistry 11*, 766–771.

Fling, S. P., & Gregerson, D. S. (1986) *Anal. Biochem. 155*, 83–88.

Glaudemans, C. P. J., Manjula, B. N., Bennett, L. G., & Bishop, C. T. (1986) *Immunochemistry 14*, 675–679.

Hochman, J., Gavish, M., Inbar, D., & Givol, D. (1976) *Biochemistry 15*, 2706–2710.

Horne, C., Klein, M., Polidoulis, I., & Dorrington, K. J. (1982) *J. Immunol. 129*, 660–664.

Huston, J. S., Levinson, D., Mudgett-Hunter, M., Tai, M. S., Novotny, J., Margolies, M. N., Ridge, R. J., Bruccoleri, R. E., Haber, E., Crea, R., & Oppermann, H. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 5879–5883.

Jaenicke, R., & Rudolph, R. (1986) *Methods Enzymol. 131*, 218–250.

Klein, M., Kortan, C., Kells, D. I. C., & Dorrington, K. J. (1979) *Biochemistry 18*, 1473–1481.

Kraut, J. (1988) *Science 242*, 533–540.

Kunkel, T. A., Roberts, J. D., & Zakour, R. A. (1987) *Methods Enzymol. 154*, 367–382.

Lerner, R. A., & Benkovic, S. J. (1988) *Bioessays 9*, 107–112.

Lesk, A. M., & Hardman, K. D. (1982) *Science 216*, 539–540.

Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Metzger, H., Chesebro, B., Hadler, N. M., Lee, J., & Otchin, N. (1971) in *Progress in Immunology: Proceedings of the 1st Congress of Immunology* (Amos, C. B., Ed.) pp 253–267, Academic Press, New York.

Pabo, C. O., & Suchanek, E. G. (1986) *Biochemistry 25*, 5987–5991.

Plückthun, A., Glockshuber, R., Pfitzinger, I., Skerra, A., & Stadlmüller, J. (1987) *Cold Spring Harbor Symp. Quant. Biol. 52*, 105–112.

Plückthun, A., Skerra, A., Glockshuber, R., & Stadlmüller, J. (1988) in *Protein Structure and Protein Engineering*, 39th Colloquium Mosbach (Winnacker, E. L., & Huber, R., Eds.) pp 123–131, Springer-Verlag, Berlin.

Plückthun, A., Glockshuber, R., Skerra, A., & Stadlmüller, J. (1989) *Behring Inst. Mitt.* (in press).

Pollet, R., & Edelhoch, H. (1973) *J. Biol. Chem. 248*, 5443–5447.

Rudikoff, S., Potter, M., Segal, D. M., Padlan, E. A., & Davies, D. R. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 3689–3692.

Satow, Y., Cohen, G. H., Padlan, E. A., & Davies, D. R. (1986) *J. Mol. Biol. 190*, 593–604.

Schultz, P. G. (1988) *Science 240*, 426–433.

Scopes, R. K. (1982) *Protein Purification: Principles and Practice*, pp 241–242, Springer-Verlag, New York.

Sedlacek, H. H., Schulz, G., Steinstraesser, A., Kuhlmann, L., Schwartz, A., Seidel, L., Seemann, G., Kraemer, H.-P., & Bosslet, K. (1988) *Monoclonal Antibodies in Tumor*

Therapy, Contributions to Oncology Vol. 32, Karger-Verlag, Basel.
Segal, D. M., Padlan, E. A., Cohen, G. H., Rudikoff, S., Potter, M., & Davies, D. R. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 4298-4302.
Skerra, A., & Plückthun, A. (1988) *Science 240*, 1038-1040.
Vieira, J., & Messing, J. (1982) *Gene 19*, 259-268.
Vieira, J., & Messing, J. (1987) *Methods Enzymol. 153*, 3-11.
Watt, R. M., & Voss, E. W. (1979) *J. Biol. Chem. 254*, 7105-7110.

Organization and Expression of the Rat $D2_A$ Receptor Gene: Identification of Alternative Transcripts and a Variant Donor Splice Site†

Karen L. O'Malley,*,‡ Kenneth J. Mack,‡ Kuan-Yun Gandelman,‡ and Richard D. Todd§
Department of Anatomy and Neurobiology and Departments of Psychiatry and Genetics, Washington University School of Medicine, 660 South Euclid Avenue, St. Louis, Missouri 63110
Received October 30, 1989; Revised Manuscript Received November 29, 1989

ABSTRACT: We have recently reported the creation of a cell line expressing D2 receptors encoded by a gene distinct from that described by Bunzow et al. [Bunzow, J. R., Van Tol, H. H. M., Grandy, D. K., Albert, P., Salon, J., Christie, M., Machida, C. A., Neve, K., & Civelli, O. (1988) *Nature 336*, 783-787]. To provide a framework for understanding structural differences between these and other G-protein-coupled receptors, the structure of the rat gene coding for the Bunzow et al. cDNA (called $D2_A$ here) was delineated. The $D2_A$ gene contains eight exons and spans at least 50 kb. Sets of oligonucleotide primers were used in combination with the polymerase chain reaction (PCR) to determine the presence of alternative transcripts within the introns. In contrast to other G-protein-coupled receptors, the $D2_A$ gene undergoes alternative RNA processing within intron 5, resulting in an insertion of 29 amino acids to the predicted 415 amino acid sequence of the $D2_A$ protein. By use of the PCR assay the relative abundance and tissue distribution of the alternative $D2_A$ transcripts (herein termed $D2_{A415}$ and $D2_{A444}$) were determined. A variant donor splice site was also identified at the end of exon 4, a GC dinucleotide instead of the canonical GT. The variant dinucleotide was also present in the mouse but not in the human $D2_A$ gene.

Dopamine receptors have been widely studied due to their proposed roles in the treatment and etiology of many neuropsychiatric disorders. Pharmacological and physiological studies have defined two principle types of dopamine receptors, D1 and D2, each with distinct pharmacological binding profiles, signal transduction systems, and sites of localization (Hamblin et al., 1984; Seeman et al., 1985; Kebabian, 1986; Stoof & Kebabian, 1984; Freedman & Weight, 1988; Enjalbert et al., 1988). Bunzow et al. (1988) have reported the cloning of a rat cDNA with the expression characteristics of a D2 receptor. This clone is a member of the G-protein[1]-coupled receptor family.

Recently we described a strategy for cloning cell surface proteins for which only radioligands are available (Todd et al., 1989). Using this technique, we isolated a cell line expressing a membrane-bound protein with the pharmacological characteristics of a D2 receptor. With polymerase chain reaction (PCR) analysis we have shown that the expressed D2 receptor is not the product of the Bunzow et al. D2 receptor gene (Todd et al., 1989). Therefore, there must be at least two genes that produce D2 receptor subtypes designated here as $D2_A$ (Bunzow et al., 1988) and $D2_B$ (Todd et al., 1989).

In order to characterize the functional and evolutionary relationships between these receptors and as the first step in developing experimental systems for studying the regulation of D2 receptor gene expression during development and differentiation, we have isolated and characterized the rat $D2_A$ gene.

Experimental Procedures

Materials. Most enzymes were purchased from Promega Biotec. Sequanase and AmpliTaq were from U.S. Biochemicals. Nylon membranes were from Schleicher & Schuell. Radionucleotides were purchased from Amersham. A λ Dash Fisher rat genomic library was obtained from Stratagene Cloning Systems.

Isolation of a Rat $D2_A$ Gene. A 15-kb phage recombinant clone encoding exons 2-8 was isolated as described (Todd et al., 1989). Fragments from this clone were used to screen a rat genomic library. Subsequent 5′ walking clones were obtained with either a T_3 or a T_7 promoter to generate end-specific RNA probes as per the manufacturer's protocols. The exon 1 containing recombinant phage was isolated by hybridization with a 96-bp fragment encoding nucleotides 1-96 reported by Bunzow et al. (1988). Labeling of probes, hybridization, and washing conditions were performed in accordance with standard methods (Feinberg & Vogelstein, 1983; Maniatis et al., 1982).

Oligonucleotides. Oligonucleotide primers were synthesized by the Protein Chemistry Facility, Washington University, on an Applied Biosystems DNA synthesizer. Oligonucleotides used for this study were derived from the rat $D2_A$ receptor cDNA (Bunzow et al., 1988) and included the following:

†This work was supported in part by National Institutes of Mental Health Grant MH45019. K.J.M. was supported by a National Institutes of Health Training Grant N507027.
*Address correspondence to this author.
‡Department of Anatomy and Neurobiology.
§Departments of Psychiatry and Genetics.

[1] Abbreviations: bp, base pair(s); kb, kilobase; G-protein, guanine nucleotide binding protein: PCR, polymerase chain reaction.

0006-2960/90/0429-1367$02.50/0